GENE CLONING STRATEGIES

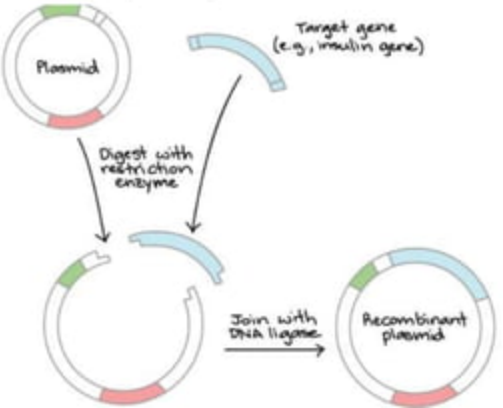
D.INDRAJA

Generation of DNA fragments

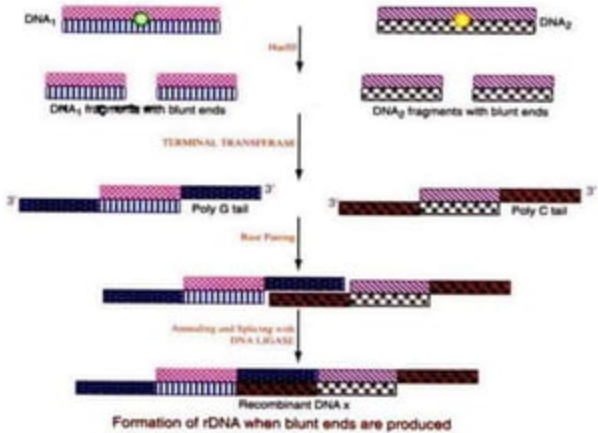
- It should be easily extractable from its source of natural existence
- The target DNA is extracted from source organism which can either be a bacterium, fungi, plant or an animal cell DNA, by various analytical methods for which the cells are first broken open to release the contents either by mechanical disruption (grinding frozen material) or by the use of chemicals like lysozyme, EDTA, the detergent-sodium dodecyl sulphate (SDS) etc., either solely or in combination with one or more chemicals.
- Then the DNA is purified from the cell extract for which the extract is treated with proteases and endonucleases and then the proteins precipitated with phenol and chloroform and finally centrifuged.
- The DNA will be measured in a spectrophotometer at 260 nm, at this wavelength the absorbance (A_{260}) of 1.0 corresponds to 50pg of double-stranded DNA/ml.
- This ultraviolet absorbance can also be used to check the purity of a DNA wherein the ratio of DNA absorbance at 260 nm and 280 nm (A_{260}/A_{280}) is 1.8. The ratio less than 1.8 indicates that the preparation is contaminated, either with protein or phenol
- It should be able to be incorporated in the vector at such a place where it can be replicated, transcribed and translated as desired.
- The gene product (protein) produced should either be commercially important or important for research purpose

• Selection of a Suitable Cloning Vector DNA

- The cloning vector is the DNA molecule into which the target DNA is introduced producing the recombinant DNA molecule. A good cloning vehicle is one which has only a single site for cutting by a particular restriction endonuclease
- To join together two duplex DNAs from different species, the two DNAs are separately acted upon by the same restriction endonuclease giving staggered (cohesive/sticky) two stranded cut.
- Therefore the staggered ends of the two DNAs will be complementary in sequence. Then the two cut DNAs are heated, mixed and cooled, so the sticky ends will base-pair to produce a new kind of recombinant DNA which is joined by DNA ligase.



- They are some restriction enzymes that produce blunt ends then
- Both the target DNA and the vector DNA are acted Upon by the same restriction endonuclease producing blunt ends.
- Poly 'G' tails are added at the 3′ ends on both strands of the target duplex DNA and poly 'C' tails at the 3′ end of the vector DNA by the use of enzyme terminal transferase.
- Since these two added tails are complementary to each other, they will enable the two DNAs to be paired with the created cohesive ends upon heating and cooling. The nicks are joined by the enzyme ligase

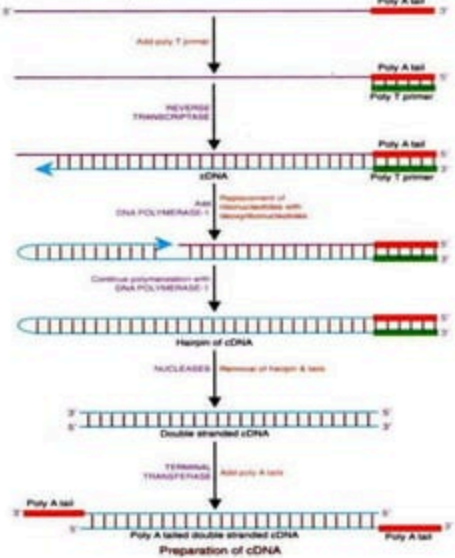


Formation of rDNA when blunt ends are produced

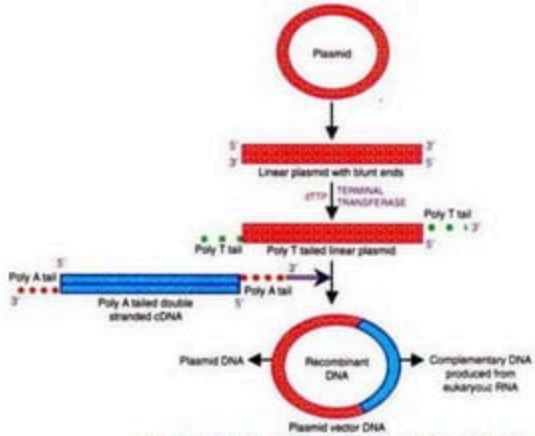
- There are different types of vectors which can be used to clone fragments of foreign DNA and propagate (clone) them in a suitable host.

Vector	Host cell
1. Plasmids	*E. Coli*
2. Bacteriophage - lambda	*E. Coli*
3 Bacteriophage M13	*E. Coli*
4. Cosmids	*E. Coli*
5. Bacteriophage QC31	Yeast
6. YE_p	Yeast
7. yIpS	Yeast and fungi
8. yRpS	Yeast and fungi
9. Ti plasmid	Plants (dicotyledons)
10. CaMv (Cauliflower Mosaic virus)	Cauliflower
11. pBR322 plasmid	Plants
12. Simian virus - 40 (SV40)	Mammalian cells
13. Bovine papilloma virus (BPV)	Mammalian cells

- **Preparation of Complementary DNA (cDNA):**
- It is a cloning technique which involves the conversion of purified mRNA to DNA, prior to its insertion into a vector. Depending upon the source of mRNA its purification procedure varies and in fact many methods for purification of mRNA are available.



Preparation of cDNA



cDNA incorporated into plasmid to form recombinant vector DNA

- **Methods of transformation of rDNA molecule In to host**
- Some commonly used procedures are as follows
- **TRANSFORMATION:**
- In rDNA technology the most common method to introduce rDNA into living cell are called transformation.
- When the DNA molecule is kept in close proximity of bacterial cells, most species of bacteria are able to take up DNA molecules from the medium without any difficulty.
- In this procedure, bacterial cells take up DNA from the surrounding environment
- However, in nature the frequency of transformation of many cells (example yeast and mammalian cells) is very less. Secondly, all the time host cells do not undergo transformation, because they are not prepared for it.
- There are some factors which affect transformation such as concentration of foreign DNA molecule, host's cell density, temperature etc.
- Some species of bacteria cannot take DNA easily hence they have to be treated physically and/ or chemically in order to make them competent to take up DNA molecules.

Physical methods

1. Microinjection
2. Biolistics transformation

Chemical methods

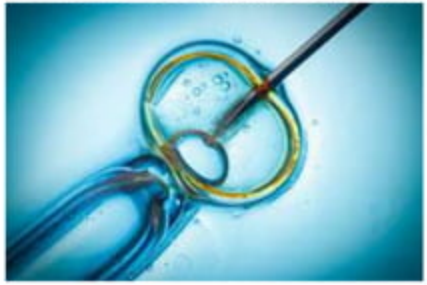
1. DNA transfer by calcium phosphate method
2. Liposome mediated transfer
3. Transfer of DNA by use of polyethene glycol
4. DNA transfer by DAE-dextran method

Electrical methods

1. Electroporation

Microinjection

- The microinjection is the process of transferring the desirable DNA into the living cell ,through the use of glass micropipette .
- Glass micropipette is usually of 0.5 to 5 micrometer, easily penetrates into the cell membrane and nuclear envelope.
- The desired gene is then injected into the sub cellular compartment and needle is removed



Limitations of microinjection

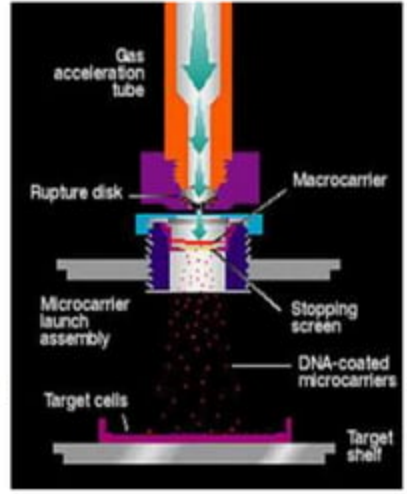
- Costly.
- Skilled personal required.
- More useful for animal cells.

BIOLISTICS

- Artificial direct method of DNA transfer
- One of the novel physical method of exogenous DNA transfer
- This physical method uses accelerated micro projectiles to deliver DNA OR other molecules into intact tissues and cells
- Also known as GENE GUN or particle bombardment
- A gene gun is a device that literally fires DNA into target cells

- **PRINCIPLE STEPS**

Step 1: the gene gun apparatus is ready to fire.
Step 2: Helium fills the chamber and pressure builds against the rupture disk.
Step 3: the pressure eventually reaches the point where the rupture disk breaks, and the resulting burst of helium propels the DNA/gold-coated macrocarrier ('Plastic Disk') into the stopping screen.
Step 4: when the macrocarrier hits the stopping screen, the DNA-coated gold particles are propelled through the screen and into the target cells.



Working

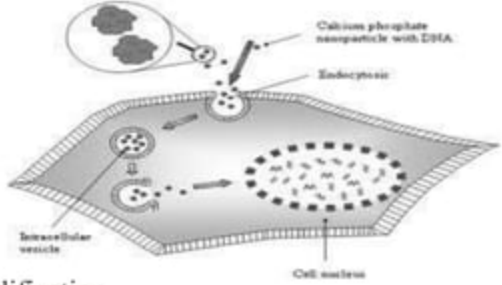
- The DNA to be transformed into the cells is coated onto microscopic beads made of either tungsten or gold
- The coated beads are then attached to the end of the plastic bullet and loaded into the firing chamber of the gene gun
- An explosive force fires the bullet down the barrel of the gun towards the target cells that lie just beyond the end of the barrel
- When the bullet reaches the end of the barrel it is caught and stopped, but the DNA coated beads continue on towards the target cells
- Some of the beads pass through the cell wall into the cytoplasm of the target cells
- Here the bead and the DNA dissociate and the cells become transformed
- Once inside the target cells, the DNA is solubilized and may be expressed

LIMITATIONs

- Integration is random
- Requirement of equipment's
- Shallow penetration of particles
- Associated cell damage
- The tissue to incorporate the DNA must be able to regenerate
- Equipment itself is very expensive

CALCIUM PHOSPHATE MEDIATED DNA TRANSFER

- The process of transfection involves the admixture of isolated DNA (10-100ug) with solution of calcium chloride and potassium phosphate under condition which allow the precipitate of calcium phosphate to be formed.
- Cells are then incubated with precipitated DNA either in solution or in tissue culture dish. A fraction of cells will take up the calcium phosphate DNA precipitate by endocytosis.
- Transfection efficiencies using calcium phosphate can be quite low, in the range of 1-2 %. It can be increased if very high purity DNA is used and the precipitate allowed to form slowly.



Limitations

- Frequency is very low.
- Integrated genes undergo substantial modification.
- Many cells do not like having the solid precipitate adhering to them and the surface of their culture vessel.
- Due to above limitations transfection applied to somatic gene therapy is limited.

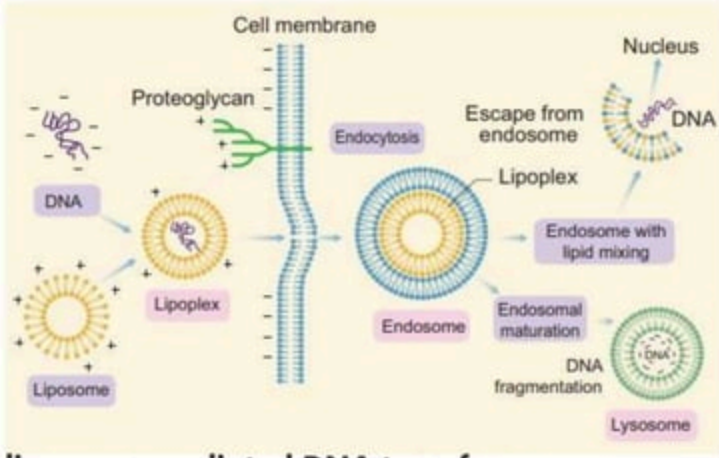
Sometimes, calcium phosphate may result in precipitate and toxicity to the cells. Some workers use diethyl amino ethyl dextran (DEAE -dextran) for DNA transfer.

DNA TRANSFER BY DAE-DEXTRAN METHOD

- DNA can be transferred with the help of DAE Dextran also
- DAE-Dextran may be used in the transfection medium in which DNA is present.
- This is polycationic, high molecular weight substance and convenient for transient assays
- It does not appear to be efficient for the production of stable transfectants.
- If DEAE-Dextran treatment is coupled with Dimethyl Sulphoxide (DMSO) shock, then upto 80% transformed cell can express the transferred gene.
- It is known that serum inhibits this transfection so cells are washed nicely to make it serum free
- Stable expression is very difficult to obtain by this method
- Treatment with chloroquinine increases transient expression of DNA.
- The advantage of this method is that, it is cheap, simple and can be used for transient cells which cannot survive even short exposure of calcium phosphate

LIPOSOME MEDIATED GENE TRANSFER

- Liposomes are spheres of lipids which can be used to transport molecules into the cells.
- These are artificial vesicles that can act as delivery agents for exogenous materials including transgenes.
- They are considered as sphere of lipid bilayers surrounding the molecule to be transported and promote transport after fusing with the cell membrane.
- Cationic lipids are those having a positive charge are used for the transfer of nucleic acid.
- These liposomes are able to interact with the negatively charged cell membrane more readily than uncharged liposomes, with the fusion between cationic liposome and the cell surface resulting in the delivery of the DNA directly across the plasma membrane.
- Cationic liposomes can be produced from a number of cationic lipids, e.g. DOTAP and DOTMA.
- These are commercially available lipids that are sold as an in vitro-transfecting agent, as lipofectin.
- Liposomes for use as gene transfer vehicles are prepared by adding an appropriate mix of bilayer constituents to an aqueous solution of DNA molecules.
- The liposomes are then ready to be added to target cells.

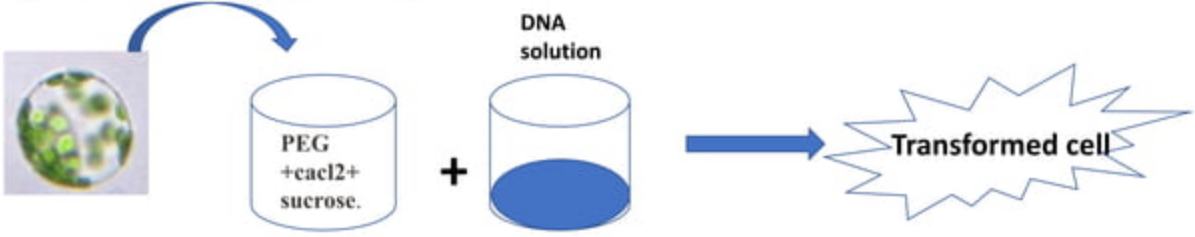


Advantages of liposome mediated DNA transfer

1. Simplicity.
2. Long term stability.
3. Low toxicity.
4. Protection of nucleic acid from degradation.

POLYETHYLENE GLYCOL MEDIATED TRANSFECTION

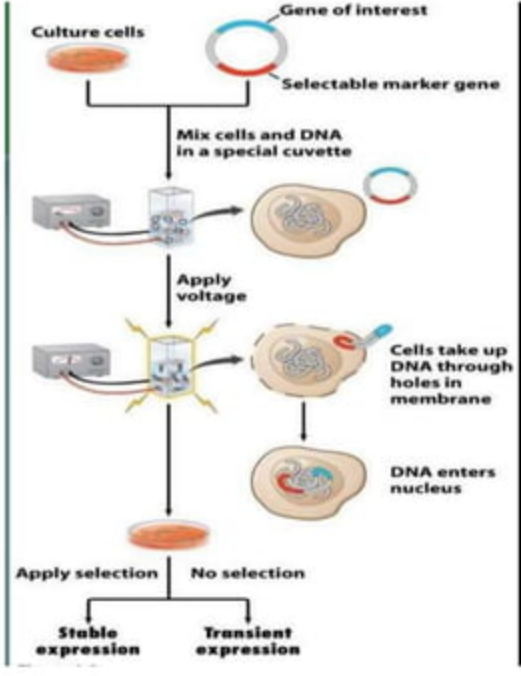
- This method is utilized for protoplast only. Polyethylene glycol stimulates endocytosis and therefore DNA uptake occurs.
- Protoplasts are kept in the solution containing PEG.
- Calcium chloride is added and sucrose and glucose acts as osmotic buffering agent.
- Polyethylene glycol (PEG), in the presence of divalent cations (using Ca^{2+}), destabilizes the plasma membrane of protoplasts and renders it permeable to naked DNA. After exposure of the protoplast to exogenous DNA in presence of PEG and other chemicals the DNA enters nucleus of the protoplasts and gets integrated with the genome.
- After several passages in selectable medium frequency of transformation is calculated. PEG based vehicles were less toxic and more resistant to nonspecific protein adsorption making them an attractive alternative for non-viral gene delivery.



- **Limitations of PEG-mediated transformation:**
- i. The DNA is susceptible for degradation and rearrangement.
- ii. Random integration of foreign DNA into genome may result in undesirable traits.
- iii. Regeneration of plants from transformed protoplasts is a difficult task

Electroporation

- Electroporation is based on the principle that high voltage electric pulses can induce cell plasma membranes to fuse.
- Thus, electroporation is a technique involving electric field-mediated membrane permeabilization.
- Electric shocks can also induce cellular uptake of exogenous DNA (believed to be via the pores formed by electric pulses) from the suspending solution.
- Electroporation is a simple and rapid technique for introducing genes into the cells from various organisms (microorganisms, plants and animals).
- The cells are placed in a solution containing DNA and subjected to electrical shocks to cause holes in the membranes.
- The foreign DNA fragments enter through the holes into the cytoplasm and then to nucleus.



Limitations of electroporation:

i. Under normal conditions, the amount of DNA delivered into plant cells is very low.

ii. Efficiency of electroporation is highly variable depending on the plant material and the treatment conditions.

iii. Regeneration of plants is not very easy, particularly when protoplasts are used.

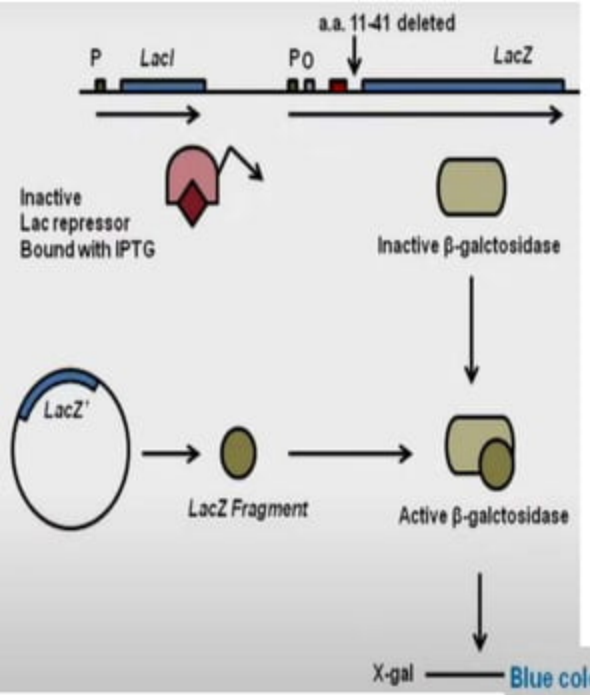
Screening and identification of recombinants

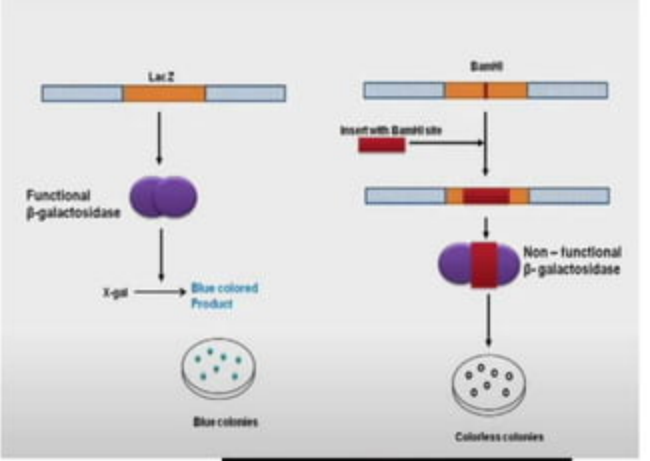
- After the introduction of rDNA in to a suitable host cell it is essential to identify those cells which have received the recombinant molecules this is called screening
- The vector or foreign DNA present in the recombinant cells express the characters while the non recombinants do not express the characters
- They are several methods to identify the recombinants some of them are based on
- Protein based
- Antibiotic based
- Nucleic acid based

Blue-White Screening

Chromogenic Substrate- The use of chromogenic substrate to detect a particular enzymatic activity is the basis to screen the desired clone.

- The most popular system to exploit this feature is "Blue white screening" where a colorless substrate is processed to a colored compound.
- The colorless compound X-gal or 5-bromo-4-chloro-3 indolyl-β-D-galactoside used in this screening method is a substrate for B galactosidase.
- The enzyme B-galactosidase is the product of lacZ gene of the lac operon. It is a tetrameric protein and an initial N-terminal region (11-41) of the protein is important for activity of the protein.
- In this system, host contains lacZ gene without the initial region where as vector contains a-peptide to complement the defect to form active enzyme.
- As a result, if a vector containing α peptide will be transformed into the host containing remaining lacz, the two fragment will reconstitute to form active enzyme.
- In addition, the peptide α region in vector contains multiple cloning site and as a result of insertion of gene fragment, consequently a-peptide will not be synthesized to give fully active B-galactosidase.
- The enzyme ß-galactosidase oxidizes X-gal to form 5-bromo-4-chloro-indoxyl and galactose.
- The indoxyl derivative is oxidized in air to give a blue colored dibromo-dichloro derivative. hence, blue colored colonies indicate the presence of an active enzyme or absence of insert where as colorless colonies indicate presence of an insert.


a.a. 11-41 deleted
P
LacI
PO
LacZ
Inactive
Lac repressor
Bound with IPTG
Inactive β-galctosidase
LacZ'
LacZ Fragment
Active β-galctosidase
X-gal
Blue colored
Product

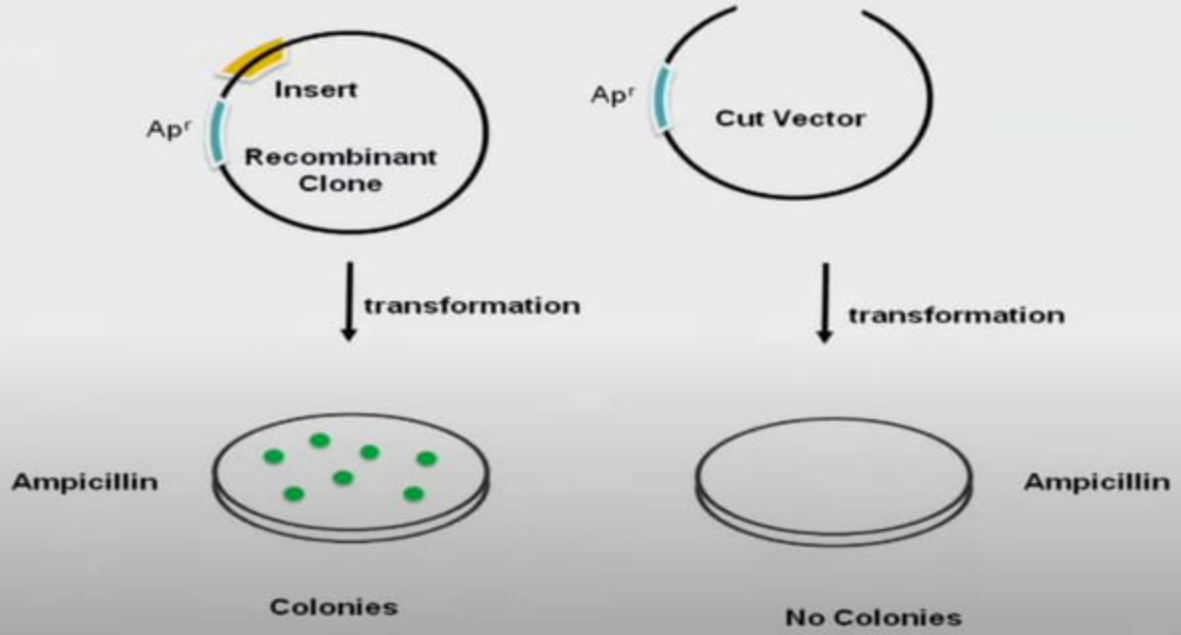

LacZ
Functional
β-galactosidase
X-gal
Blue colored
Product
Blue colonies
BamHI
Insert with BamHI site
Non – functional
β- galactosidase
Colorless colonies

Antibiotic sensitivity

- Vector carries a functional selection marker such as antibiotic resistance gene to be use to select the clone.
- The antibiotic resistance gene product has multiple mechanism to provide resistance in host cell.
- In this approach, a circular plasmid containing antibiotic resistance can be able to replicate into the host cell plated on a antibiotic containing media.
- In the cloning of a fragment into this plasmid, the plasmid is cut with restriction enzymes and a fragment is ligated to give circular plasmid with insert.
- The transformation of both DNA species; cut plasmid and circularized clone into the host and plated onto the antibiotic containing solid media.
- Only circularized clone will give colonies where as cut plasmid will not grow as it has lost antibiotic resistance gene.

TABLE: ANTIBIOTICS RESISTANCE GENE AND THEIR MODE OF MECHANISM.

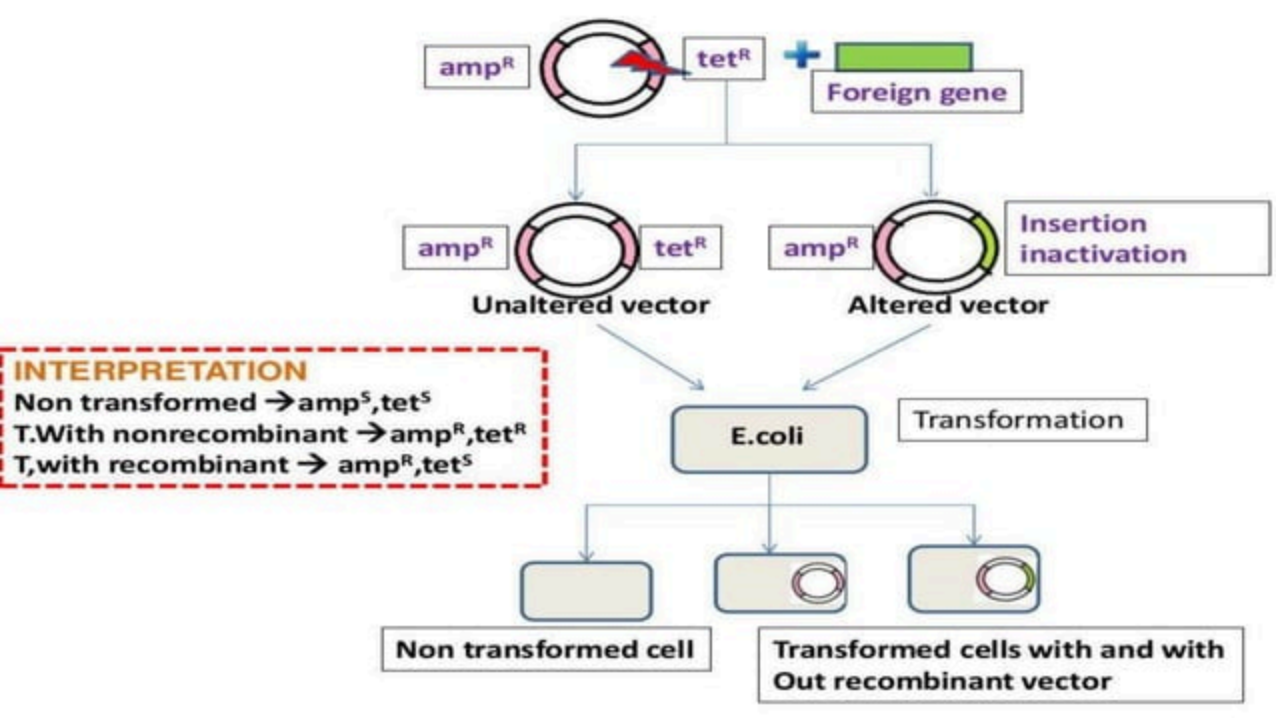
Antibiotic	Gene product	Mechanism
Ampicillin	β-lactamase	Degradation of ampicillin
Kanamycin	Neomycin phosphotransferase II	Covalent modification of kanamycin
Tetracycline	Ribosomal protection proteins	Efflux of tetracycline outside of the bacteria
Chloramphenicol	Chloramphenicol acetyl transferase	Chloramphenicol to acetyl Chloramphenicol


Insert
Ap^r
Recombinant Clone
Ap^r
Cut Vector
transformation
transformation
Ampicillin
Ampicillin
Colonies
No Colonies

Insertional inactivation

- In this approach a foreign DNA is cloned with in the coding gene responsible for a phenotype. As a result of insertion the gene product is not available to modulate the phenotype of the host. This approach is known as insertion inactivation and it can be used with a suitable genetic system
- It is a technique used in recombinant DNA technology to select recombinant colonies
- It involves the inactivation of a gene up on insertion of another gene inside in its place or with in its coding sequence
- For example-pBR322 is opened by using a restriction enzyme BamHI where restriction site lies with in the tetracycline resistant gene.
- The foreign DNA also isolated with the same type of restriction enzyme and DNA ligase is added to the mixture containing linearized pBR322 and the foreign gene two types of vectors are formed
- Recombinant vector → foreign gene ✓
- Un altered vector → foreign gene ✗

- When this mixture of both altered(recombinant)and unaltered vector are used for transformation of E.coli two types of cells maybe formed
- NonTransformed cells → With altered vector
- Transformed cells → With unaltered vector
- Both the cells are transferred to ampicillin containing media then
- Transformedcell →live
- Nontransformedcell →dead
- Now the only transformed cells are transferred to ampicillin containing media and this is considerd as masterplate
- To identify recombinant cells replicate plate is prepared containing tetracycline media in this
- Transformed with recombinant → dead as insertional inactivation happened
- Transformed with nonrecombinant → live as it is resistant to both antibiotics
- Now by comparing with the master plate recombinant transformed cells are identified


amp^R
tet^R
Foreign gene
amp^R
tet^R
Unaltered vector
amp^R
Insertion inactivation
Altered vector
INTERPRETATION
Non transformed → amp^S, tet^S
T.With nonrecombinant → amp^R, tet^R
T,with recombinant → amp^R, tet^S
E.coli
Transformation
Non transformed cell
Transformed cells with and with Out recombinant vector

Non transformed cell

Transformed cells with and with Out recombinant vector

INTERPRETATION

Non transformed → amp^S, tet^S

T.With nonrecombinant → amp^R, tet^R

T,with recombinant → amp^R, tet^S

Amp

Non transformed cells → dead

Transformed cells → live

Transformed cells are transferred

Amp

Master plate

tet

Transformed with recombinant → dead

Transformed with unaltered → live

Replica plate

Compared with the master plate and recombinants are picked

Nucleic acid hybridization

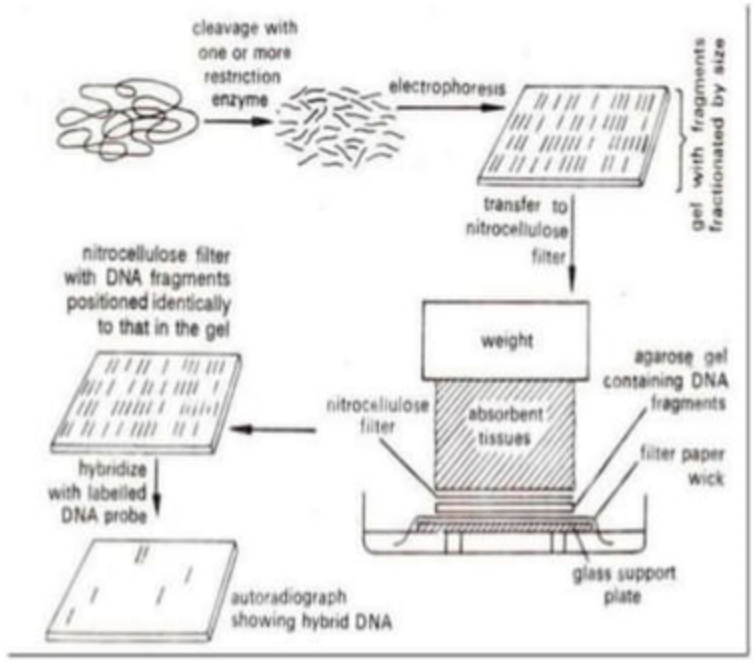
- A technique in which single-stranded nucleic acids (DNA or RNA) are allowed to interact so that complexes called hybrids are formed by molecules with similar, complementary sequences.
- Through nucleic acid hybridization, the degree of sequence identity between nucleic acids can be determined and specific sequences detected in them. The hybridization can be carried out in solution or with one component immobilized on a gel or, most commonly, on nitrocellulose paper
- **Nucleic acid blotting technique**: Blotting refers to process of immobilization of sample nucleic acid in solid support.
- The blotted nucleic acids are then used as target in the hybridization experiment for their specific detection.

Types of blotting techniques:

- Southern blotting
- Northern blotting
- Colony blotting
- Dot blotting

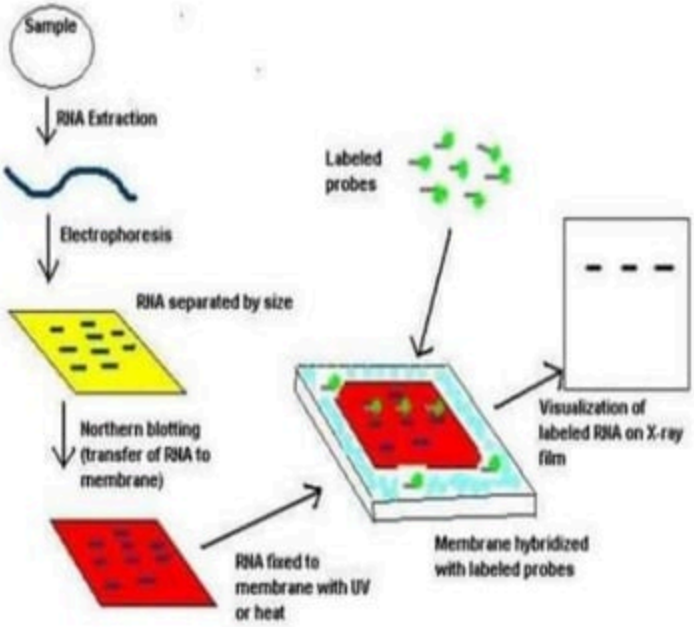
Southern blotting

- Southern blot is a techniques employed for detection of a specific DNA sequence in DNA samples that are complementary to a given RNA or DNA sequence.
- It was first given by E.M Southern, a British biologist.
- This methods includes separation of restricted DNA fragments by electrophoresis and then transferred to a nitrocellulose or a nylon membrane, followed by detection of the fragment using probe hybridization.



Northern blotting

- Northern blotting was developed by James Alwine, George stark and David Kemp (1977).
- In this technique, RNA is being analysed instead of DNA.
- It is a technique by which RNA fragments are separated by electrophorosis and immobilized on a membrane
- The identification of specific RNA is done by using nucleic acid probes. It helps to study gene expression by detection of RNA.

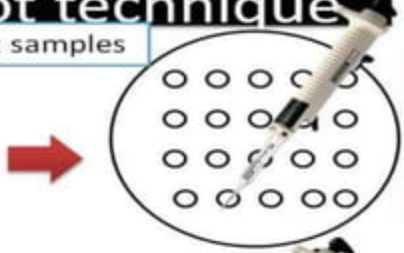


Dot blot

- This technique is used to detect the presence of a given sequence of DNA/RNA in the non-fractionated(not subjected to electrophoresis) DNA sample
- DNA from many samples can be tested in a single test.
- A Dot blot (or Slot blot) is a technique used to detect biomolecules
- It represents a simplification of the northern blot, Southern blot, or western blot method
- In a dot blot the biomolecules to be detected are not first separated • Instead, a mixture containing the molecule to be detected is applied directly on a membrane as a dot
- Then is spotted through circular templates directly onto the membrane or paper substrate.
- Then followed by detection by either nucleotide probes (for a northern blot and Southern blot) or antibodies (for a western blot).
- It offers no information on the size of the target biomolecule. Furthermore, if two molecules of different sizes are detected, they will still appear as a single dot.
- Can only confirm the presence or absence of a biomolecule.

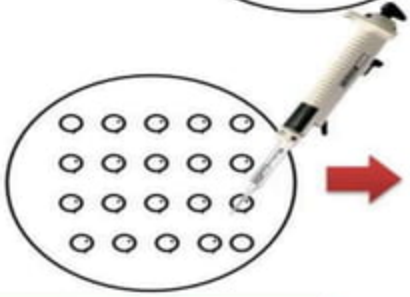
STEPS in Dot blot technique

S 1: Purified DNA/RNA of different samples

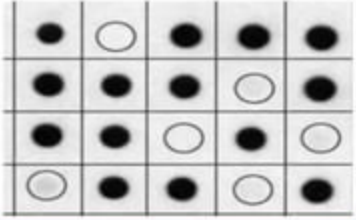


S 2: Apply directly as small dots on nitrocellulose or nylon membrane
*Denature for DNA
Immobilize by baking at 70-80 C for 2-3 H

S 3: Add labeled probe for hybridization & incubate: Probe binds to the complementary strand forming dsDNA



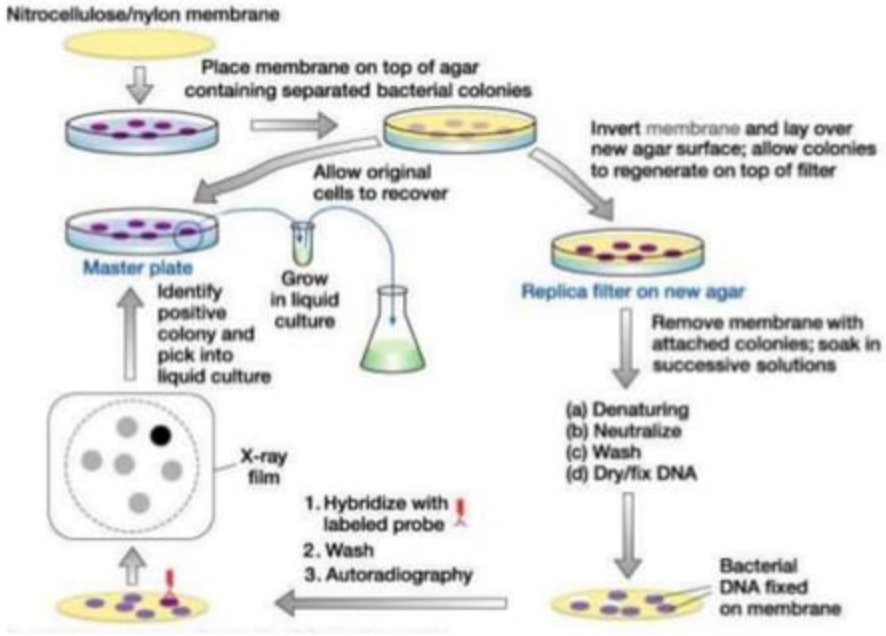
S 4: Wash off excess un bound probes



S 5: Autoradiograph
Black dots indicate the presence of sequence in that samples

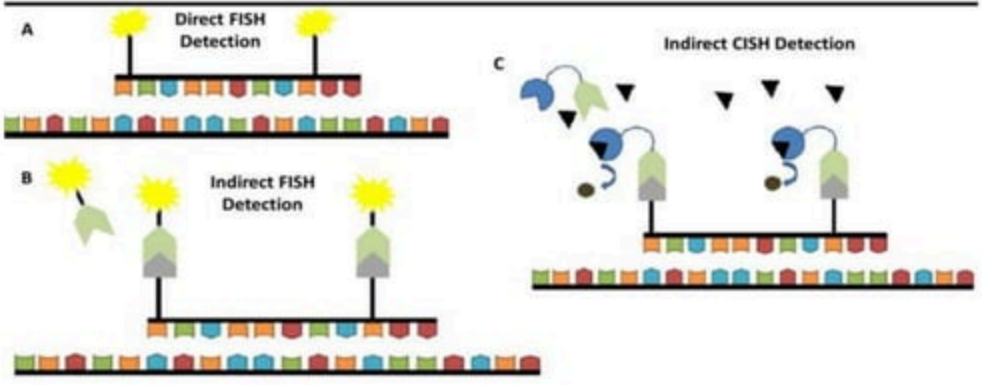
Colony hybridization

- Colony hybridization is used to identify bacterial colonies containing the gene of interest Colonies are transferred to filter, lysed, DNA is denatured by high pH
- Membrane is then hybridized with labeled DNA from gene to be cloned



In situ Hybridization

- It is a technique that employs a labeled complementary nucleotide strand for localizing specific DNA or RNA sequence targets within fixed tissues and cells.
- There are two ways to detect DNA or RNA targets:
- i) Chromogenic insitu hybridization
- ii) Flourescence insitu hybridization.




ThaNK
YOU